Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2014 Vol. 4 (2) April-June, pp.596-602/Akbari et al.
Research Article

EVALUATING THE MICROPROPAGATION OF LISIANTHUS (*EUSTOMA GRANDIFLORA* L.) AS AN IMPORTANT ORNAMENTAL PLANT

***Hassan Akbari[1], Reyhaneh Pajooheshgar[2] and Narges Karimi[3]**
[1]*Agricultural Research and Natural Resources of Esfahan, Esfahan, Iran*
[2]*Student University of Mashhad, Departments of Horticulture, Mashhad, Iran*
[3]*University of Esfahan, Departments of Biotechnology, Esfahan, Iran*
**Author for Correspondence*

ABSTRACT
Lisianthus (*Eustoma grandiflorum* L.) is an ornamental plant with beautiful flowers. That is a very popular ornamental plant. Due to low germination of its seeds, usual techniques for its propagation are not efficient. This present study was aimed to investigate the effective of different media and growth regulators for *in vitro* culture of Lisianthus through indirect and direct regeneration. The results showed that indirect regeneration, callus were raised aseptically by placing surface sterilized leaf explants on B5 medium supplemented with 1.5 mg/L NAA in (of 115 µMs-1m^2 at the flask level) lux light 16/8 h photoperiod, 25±2 ºC conditions. Shoot formation was derived from callus in B5 medium containing 1.5 mg/L KIN+ 0.5 mg/L GA3. Results show rootlet formation in 1.5 mg/L NAA using B5 medium. For direct regeneration, the most effective proliferation of axillary buds was observed in B5 medium supplemented with 1 mg/L GA3+1 mg/L BAP.

Keywords*: Callus, Eustoma Grandiflorum L., Regeneration, Axillary Buds.*

INTRODUCTION
Lisianthus (*Eustoma grandiflorum* L.) is an ornamental, herbaceous annual plant from Gentianaceae. The plant grows to 15 - 60 cm tall, with bluish green, slightly succulent leaves, mature rapidly, and produce beautiful funnel shaped flowers growing on long straight stems. Lisianthus which is one of the newest ornamental plants in international market because of rose-like flower and vase life among 10 top cut flowers in the world (Kunitake *et al.,* 1995; Rezaee *et al.,* 2012). The plant is well known for its long vase life, size, and different colors of its flowers (Hecht *et al.,* 1994). In addition, the plant is tolerant to pathogen, soil acidity condition, and high temperature stresses. However, because of small size of the Lisianthus seeds (19,000 seed /gm or 545,000 seeds /oz) it is hard to handle in field plantings. Traditional method of Lisianthus propagation is by cutting, which is considered as slow method and by seed that produces seedling which is not true to the type (Evans and Bravo, 1985; Popa *et al.,* 2006). *In vitro* culture technique can be effectively used to over comes these barriers. Also micro propagation is an important asexual method that can be used for the production of virus-free plants. In addition, more research has been devoted to the investigation and improvement of Lisianthus by genetic engineering, such as regulation of floral transition (Zaccai *et al.,* 2001; Zaccai and Edri, 2002; Pack *et al.,* 2000), or production of Lisianthus flowers with altered pigment metabolic pathways (Aranovich *et al.,* 2007). The shoot tips explants from Lisianthus were cultured on MS medium supplemented with concentrations of 0, 0.5, 1 and 2 mg/L of NAA and KIN. Here, we present a simple and reliable strategy for micro propagation of Lisianthus in resented of the single growth regulator, KIN, which enables the production of stock plants. Multiple shoots containing roots can be obtained simultaneously on MS basal medium only supplemented with 0.5-1 mg/L KIN. Also, the most number of nodes per explants (8.86) was obtained in medium containing 0.5 mg/L KIN without NAA. The highest root number per shoot (2.40) was seen in medium supplemented with 2 mg/L KIN + 0.5 mg/L NAA. The results of this study revealed that the best shoot proliferation was achieved in MS medium supplemented with 0.5 or 1 mg/L KIN without NAA. Regenerated plants were transferred to peat and prelate (1:1) after hardening and they showed 100% survival (Ghaffari *et al.,* 2012). Results showed that among different plant parts, leaf explants were pioneer to produce callus. Basal LS medium containing 3 mg/L IAA, 3 mg/L NAA, 0.1

mg/L kinetin, and B5 medium containing 0.225 mg/L BA and 1.86 mg/L NAA were the best media for induction of callus. The calluses in LS media started for organogenesis (rhizogenesis) earlier than those in B5, and MS medium containing 3 mg/L IAA, 3 mg/L NAA, and 2 mg/L Glycin (Rezaee, *et al.*, 2012).This study was conducted to identify the best type of explants from *in vitro* grown seedling and growth regulator concentration and combination for callus induction, shoot regeneration. The objective of the present study was to evaluate the effects of different concentrations of KIN and NAA on regeneration of shoot and root in Lisianthus from callus, direct shoot regeneration from axillary buds and rooting of regenerated shoots, from different tissues part by using *in vitro* techniques.

MATERIALES AND METHODS

Plant Material

Explants of Lisianthus were collected from one year old seedling in the green house at Mashhad University of Iran. Segments of young leaves, internodes, and roots (2–4 mm in diameter) were obtained from one year old rooted cuttings. The samples were surface sterilized by subsequent washing with detergent, sodium hypochlorite (containing 5% active chlorine, 20 min), ethanol (75%, 30 s) with rinsing in sterile distilled water intervals. Surface sterilization was accomplished under laminar air flow. The explants were aseptically cut into small pieces. Axillaries buds with at least 2 leaves, young leaves with the size 0.6× 0.6 cm, stem segments with the size 1 cm and tip roots were placed on different mediums supplemented with different combinations of hormones. To remove probable contaminations from root samples, they were washed with potassium permanganate (2%, 3 min) before washing with detergent.

Medium

Medias consisting MS (Murashig–Skoog 1962) and B5 (Gamborg *et al.*, 1965), 1.5% sucrose were provided. Different hormones added to media. A volume of 25 ml of nutrient medium was dispensed into 3 flasks that were capped with a polystyrene screw cap. The pH of media was adjusted to 5.7 with 0.1N NaOH or 0.1N HCl prior to autoclaving at 1.05 kg/cm², 121°c for 20 min.

Culture Media

For callus induction, sterile explants of leaves, stem segments, tip roots were aseptically placed on B5 medium supplemented with different combination of 2,4- D (0- 1-1.8 mg/l) and NAA (0 -1- 1.5 mg/l), containing 25 g/l (w/v) of sucrose and 8 g/l (w/v) agar. The explants were incubated at 25±2 °C under dark and light (flux of 115 µMs-1m2 at the flask level) condition. After 10 weeks of culture, the percentage of explants producing callus was recorded. The observation was made on the morphology of callus formed in different phytohormones tested.

In vitro shoot Induction

Well-proliferated calli derived from the leaf segments 7 weeks after culture were used for regeneration studies. Approximately 1 grams of fresh callus was placed on the B5 medium containing 1.5 mg/l BAP + 0.5 mg/L NAA or IAA or GA_3 and 1.5 mg/l K + 1 mg/L IAA or NAA or GA_3. Data on the mean number of transferable shoots per explants and percentage of shoot formation and shoot length were observed after 16 weeks of culture. Each experiment consisted of 3 replicates. The cultures were incubated at 25±2 °c under an illumination of flux of 115 µMs-1m^2 at the flask level during a 16/8 h photoperiod obtained from fluorescent lamps.

In vitro Rooting Induction

For induction of roots, in vitro regenerated shoots were individually transferred to B5 basal medium supplemented with 25 g/L (w/v) of sucrose and 8 g/L (w/v) of agar and plant growth regulators. Plant growth regulator supplied in this experiment was NAA (0 -1- 1.5 mg/L) and 2, 4- D (0 -1- 1.5 mg/L). The frequency of rooting average root number per shoot and root length was recorded. All cultures were incubated at 25±2 °C under 16/8 h photoperiod.

Proliferation Axillary's Buds

Stem segments with axillary's buds cultured on medium consisting, MS and B5 medium. Plant growth regulators applied in this experiment were 1 mg/L BAP, 1 mg/L GA3, 0.5 mg/L BAP+ 1mg/L GA3 and 1 mg/L BAP + 1 mg/L GA3. Explants were placed horizontally on the nutrient medium and were embedded

to a depth of about one – third of thickness. After 4 weeks number of shoot per explants, length shoots, percentages of shooting were recorded and photosynthetic photon flux of 115 μMs-1m^2 at the flask level.

Statistical Analysis

The factorial design based on completely random design (CRD) was used to examine the effects of culture media and type of explants Data processing of the results was carried out by an EXCEL. Analysis of variance (ANOVA) was done using MSTATC statistical software and means were compared using Duncan's test.

RESULTS AND DISCUSSION

Callus Induction

We studied the effect of different concentrations of NAA (0 - 1.5 mg/L) and 2, 4-D (0 - 1.5 mg/L), under dark and light (flux of 115 μMs-1m2 at the flask level) conditions on micro propagation of Lisianthus, an ornamental plant. Studied characteristics were shoot length, shoot number, node number, and root number. The results are summarized in Table 1. Our data revealed that there are no significant in the effect of the different concentrations and interaction between NAA and 2, 4-D regulators on these characters. But, which were significant in different concentrations of NAA. The result showed that indirect regeneration Callus was optimally induced from leaf segments derived from one year old seedling within 32 – 36 days in darkness and 24 – 28 days in light of incubation in all treatments containing NAA. Callusing was initiated at the middle of explants and eventually extended all over explants. The callus which induced from leaf explants on B5 basal medium containing NAA in dark condition was yellow and friable whereas those in light condition were green and friable. As the results were shown (Table 1), 1.5 mg/L NAA was found to produce significantly higher callus in comparison to other treatments. The result indicated that the light appeared to have an effect on growth of callus. It was observed that the fresh weight of callus by the end of incubation period in the light was higher than fresh weight of callus incubated in the dark condition. Similar result on the effect of light on callus growth was observed in the culture of *Cistanche deserticola* L. where the presence of light was found to increase the production of callus (Ouyong *et al.*, 2003). The increase of callus growth in the light condition might be related to the rate of nutrient uptake which was found higher than those in dark condition (Muse, 1989). However the results tend to contrast with observation in callus culture of *azarichta indica* L. where the maximum fresh weight of callus incubated in dark were 61% and 64% higher compared to those incubated in light condition (Simoh, 1997, Da *et al.*, 2003). Callusing wasn't occurred in all treatments containing different concentrations 2, 4-D (0-1.5mg/L). Although, auxin is able to promote the growth of callus, however according to Wernicke and Mikovitis (1987), high concentrations of 2, 4-D was able to inhibit callusing of basal segments and it had effect as the herbicide.

Table 1: Percentage callus induction, fresh weight, morphology of callus, days of callus form, from Lisianthus in medium supplemented with different levels of NAA after 4 weeks of culture in darkness and light

Darkness &light	Hormonal combination(mg/l)	Callus induction (%)	Days of callus induction	Callus fresh weight (gr)	Morphology of callus
Darkness	0 NAA	-	-	-	-
Darkness	0.5 NAA	95	27±1	0.04367f	yellow/friable
Darkness	1 NAA	97	24±2	0.1292e	yellow/friable
Darkness	1.5 NAA	100	25±2	0.2046d	yellow/friable
Light	0 NAA	-	-	-	-
Light	0.5 NAA	97	34±1	0.3348c	Green/friable
Light	1 NAA	98	35±2	0.5325b	Green/friable
Light	1.5 NAA	100	33±1	0.6507a	Green/friable

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's test

Shoot Regeneration

Callus derived from leaf explants, were used for shoot regeneration. Regeneration of green shoots buds occurred in this callus after 8 weeks of culture and maximum number of shoot bud emergence occurred in the 12 weeks. These shoot buds elongated and attained on average height of 3.2 cm in B5 medium containing 1.5 mg/L K +0.5 mg/L GA3(Skoog and Miller 1962), demonstrated contrast between auxines and cytokinines. Kinetine is more effective than Adenine, because more shooting were observed in 10^{-6} gr/L kinetine in comparison to $3*10^{-8}$ gr/l auxin, so there is a weight relation about 1:30 between 2 complexes, however about adenine this ratio must be about 1:1500. Hence, exogenous Auxin was needed for multiple shoot formation of Lisianthus, the role of BAP and K seems essential for regeneration. All the calluses cultured in B5 medium with NAA+BAP or NAA+K, did not induce shoot from explants and callus were necrosis during 20 days. Tissue necroses might be made due to high concentrations of Auxin and low concentration of Cytokinin. Similar results were obtained in Lisianthus by using NAA and BA (Semmeniuk, 1987; Rezaee, 2012).

Table 2: Percentage of shoot induction (length, No. of shoot, days of shoot regeneration) from Lisianthus in medium supplemented with different levels of GA3, NAA, BAP ,KIN, IAA after 8 weeks of culture

Hormone combination In B5 basal medium (mg/l)	Shoot regeneration (%)	Days of shoot regeneration	Shoot length(cm)	
0.5 NAA+K 1.5	-	-	-	-
0.5 IAA +1.5 K	10	44 ±1	0.11 c	1.22 c
0.5 GA_3+1.5 K	80	39±1	1.10 b	4.6 b
0.5NAA +1.5BAP	-	-	-	-
0.5IAA +1.5BAP	15	42±2	c 0.16	1.33 c
0.5GA_3 +1.5BAP	98	39±2	a 2.43	7.2 a

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's test

Rooting of the Regenerated Shoots

Result in the rootlets induction of *in vitro* plantlets derived from shoot explants cultured in B5 medium supplemented with different levels of NAA (0-1.5 mg/L) and 2,4-D (0 - 1.5 mg/L) were summarized in table 3. The data obtained reveled that only treatments with NAA managed to trigger the formation of rootlets from *in vitro* plantlets within 20-22days. Other treatments tested with 2, 4-D didn't show any sign of rootlets formation. The best development of rooting was observed on medium containing 1.5 mg/l NAA. Presence of 0.5 mg/l NAA improved root length as compared to the control. Explants response for rooting was higher than control in presence of 1mg/l NAA. Concentration and type of Auxin used markedly influenced number of roots and length of them. The results showed that Auxin is essential to induce rooting in Lisanthus as a few rooting was observed in the absence of Auxin. Further, it has been reported by Blackseley chaldecott (1997) that differentiating cell requires the most appropriate Auxin to become competent to respond to organogenesis signal. It is also clear from the result that rooting percentage showed the tendency to increase with increasing concentration of NAA. If the concentrations of NAA were raised from 0.5 mg/l to 1.5mg/l, there was increase in number and length of roots. Also Ameena (2009) showed increasing of number and length root *in vitro* cultures of *Ficus anastasia* was due to the increasing of NAA concentration and higher concentration more than 1.8 was leading to deduction of them. The inferior effect of NAA on the root number may be due to the reason that NAA is more persistent than IBA, and remains present in the tissue and may block further development of root Meristemoid (Nanda *et al.*, 2004). In the other hand, different media have different effect on plant cell due to presence of type and different amount of nutrient composition. The result showed that root forming ability was higher on B5 medium compared to MS. High level of NH^{4+} ion may be toxic to the cell as uptake of accompanying release of an H^+ equivalent, which lowers pH. High NH^{4+} level could also

depress calcium, magnesium and potassium uptake which in turn restrict the nutrient flow from medium into cell and may cause low cell yield (Omar *et al.*, 1992; Rezaee, 2012).

Table 3: Percentage root regeneration (length, No, days of root to form) from Lisianthus in medium supplemented with different levels of 2.4-D and NAA after 4 weeks of culture

Basal medium	Hormone combination	Root regeneration%	Days of root to form	Root length	No. of root
MS	0 NAA	100	22±2	0.25e	11.67f
MS	0.5 NAA	100	21±2	2d	33d
MS	1 NAA	100	10±1	3.2b	41.33b
MS	1.5 NAA	100	22±2	6.05a	56.33a
B5	0 NAA	100	21±2	0.42d	17.23e
B5	0.5 NAA	100	20±2	1.6d	35.66c
B5	1 NAA	100	22±2	2.77c	40.16b
B5	1.5 NAA	100	20±1	4.97a	59a

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's test

Table 4: Percentage shoot regeneration, shoot length, No. of shoot, days of shoot regeneration, from Lisianthus in medium supplemented with different levels of BAP and GA3 after 4 weeks of culture

Basal medium	Hormonal combination(mg/l) GA3 BAP	Shoot regeneration	Days of shoot to form	Shoot length (cm)	No. of shoot
B5	0 1	100	18±2	1.51 a	0.53 d
B5	0.5 1	100	16±2	1.58 b	1.28 a
B5	1 1	100	16±1	1.11 b	1.22 a
B5	1 0	100	17±2	0.48 c	0.76 c
MS	0 1	100	19±1	1.50 a	0.62 d
MS	0.5 1	100	18±2	0.59 c	0.98 b
MS	1 1	100	18±1	0.97 b	0.98 b
MS	1 0	100	18±2	0.47 c	0.48 d

In each column, means with the similar letters are not significantly different at 5% level of probability using Duncan's test

Figure 1: Different stages of induction of callus and regeneration of plantlets of Lisianthus. (a,b) The callus induced in B5 and MS media, respectively.

Proliferation from Axillary Buds

The concentration and type of Cytokinins used markedly influenced number and length of shoots. Also Cytokinins make cell division, shoot formation and morphogenesis. The result showed that Cytokinine is essential to induce shooting in Lisanthus micro cutting. As shown in table 4 between two media tested,

the average rate of plantlet regeneration was the highest on B5 medium containing 1 mg/L BAP + 1 mg/L GA_3. According to the results, maximum number of shoots per explants observed in medium supplemented with 1 mg/l BAP and highest length of shoots were appeared in medium containing 1 mg/L GA_3. Increasing the concentration of BAP and decreasing of apical dominant, was the reason for decreasing the length of shoots. In the other hands, GA_3 increase the length of shoots. Treatment which supplemented with two types of Cytokinin was more effective. These results are in agreement with the previous reports on Alestromeria by Khaleghi (Khaleghi, 1387). These results were similar to Rout whose reported same conclusion on multiplication in Rose (Rout *et al.*, 1992).In addition, type of medium had influence on the number and length of shoots. B5 is better than MS medium; it seemed that the slower growth obtained with the MS medium could be due to the high contents of ammonium salts, which have been reported as growth inhibitors for some plant tissue cultures (Eknankul and Ellis, 1958).

REFERENCES

Ameena AH, Maleki SA and Khaled M (2009). Effect of medium strength and charcoal combined with IBA and NAA on root of *Ficus anastasia*. *Academic Journal of Plant Sciences* **2**(3) 169-172.

Da KD, Zhang S, Zang Y, Zhang Z and Wu L (2003). Study on adventitious shoot regeneration and micro-propagation from leaves of Lisianthus (*Eustoma grandiflorum* L.). *Journal of Shandong Agricultural University* **04** (In Chinese, Abstract in English).

Eknankul D and Ellis BE (1985). Effect of auxins and cytokinins on growth and rosmarinic acid formation in cell suspension cultures of *Archuisa officinals. Plant Cell Reports* **4**(2) 50-53.

Esizad SG, Kaviani B, Tarang A and Zanjani SB (2012). Micropropagation of lisianthus (Eustoma grandiflorum), an ornamental plant. *Journal of Plant Molecular Biology & Omics* **5**(3) 314.

Evans DA and Bravo JE (1985). Phenotypic and genotypic stability of tissue cultured plants. Dordrecht, the Netherlands: Martinus Nijhoff; 73–94.

Khaleghi A, khalighi Ahmad and Azadi P (2008). Investigation of plant regeneration from alestromeria **39**(1) 39-47.

Kunitake H, Nakashima T, Mori K, Tanaka M and Mii M (1995). Plant regeneration from mesophyll protoplasts of lisianthus (*Eustomagrandiflorum*) by adding activated charcoal into protoplast culture medium. *Plant Cell, Tissue and Organ Culture* **43** 59–65.

Muse R (1989). Physiology and Biochemistry of Witches Broom Disease in coca (Theo lorona cocoal.). Ph.D Thesis, University of Liverpool, England, U.K.

Nanda RM, Das P and Rout GR (2004). *In vitro* clonal propagation of *Acacia mangium* and its evaluation of genetic stability through RAPD marker. *Annals of Forest Science* **61** 381-386.

Omar MS, Hameed MK and Rawi MS (1992). Biotechnology in Agriculture and Forestry. Protoplast and Genetic Engineering. Berlin:Bajaj , Y. P. S.Roh, S. M., Lawson, R. H. The culture of lisianthus. Greenhouse Manager 2:103–121.

Paek KY and Hahn EJ (2000). Cytokinins, auxins and activated charcoal affect organogenesis and anatomical characteristics of shoot-tip cultures of lisianthus [*Eustoma grandiflorum* (RAF.) Shinn]. *In Vitro Cellular and Development Biology - Plant* **36**(2) 128-132.

Popa G, Cornea CP and Brezeanu A (2006). Influence of different agrobacterium rhizogenes Strains on hairy roots induction in *Eustoma grandiflorum.* Available: http://ebooks.unibuc.ro/biologie/RBL/Archive/2006/nr1/lucr_9_cornea.doc.

Rezaee F, Ghanati F and Yusefzadeh Boroujeni L (2012). Micropropagation of Lisianthus (Eustoma grandiflora L.) from different explants to flowering onset. *Iranian Journal of Plant Physiology* **3**(1) 583 - 587.

Rout GR, Debata BK and Das P (1992). *In vitro* regeneration of shoots from callus cultures of Rosa hybrida L *cv Landora* Indian. *Journal of Experimental Biology* **30** 15 –18.

Semeniuk P and Griesbach RJ (1987). In vitro propagation of *Prairie gentian*. *Plant Cell, Tissue and Organ Culture* **8**(3) 249-253.

Serdang SSF and Miller CO (1957). Chemical regulation of growth and organ formation in plant tissue culture *in vitro*. *Symposia of the Society for Experimental Biology* **11** 118-181.

Simoh S (1997). Penghasilan Sebatian Bioktif Azadricachta Mentah Danpada Kutur Kalus Sel Ampaian Azadricachta A. Jus. (Mambu). MSc Thesis, University Putra Malaysia,

Uyang J, Wang X, Zhoo B and Wang Y (2003). Light intensity and spectral quality influencing the callus growth of *Cistanene deserticola* and biosynthesis of phenylethanoid glycosides. *Plant Science* **165**(3) 657-661.

Wernicke W and Mikovits I (1984). Developmental gradients in wheat leaves-response of leaf segments in different genotype cultured *in vitro*. *Journal of Plant Physiology* **115** 49-58.

Zaccai M and Edri N (2002). Floral transition in Lisianthus (*Eustoma grandiflorum*). *Scientia Horticulturae* **95** 333–340.